VIROLOGY 227, 493–499 (1997)
ARTICLE NO. VY968323

SHORT COMMUNICATION

Agglutination of Erythrocytes from Different Animal Species

TOSHIHIRO ITO,*,[1] YASUO SUZUKI,† LYNDON MITNAUL,‡ ANGELA VINES,‡ HIROSHI KIDA,* and YOSHIHIRO KAWAOKA‡,§

*Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan; †Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Science, Shizuoka 422, Japan; ‡Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, P. O. Box 318, Memphis, Tennessee 38101-0318; and §Department of Pathology, University of Tennessee, Memphis 800 N. Madison Avenue, Memphis, Tennessee 38163

Received January 2, 1996; returned to author for revision March 11, 1996; accepted October 29, 1996

Despite their uniform ability to bind to oligosaccharide-containing terminal sialic acids, influenza A viruses show differences in receptor specificity. To test whether agglutination of erythrocytes from different animal species could be used to assess the receptor specificity of influenza A viruses, we determined the agglutinating activities of a range of virus strains, including those with known receptor specificities, using erythrocytes from seven animal species. All equine and avian viruses, including those known to recognize *N*-acetyl and *N*-glycolyl sialic acid linked to galactose by the α2,3 linkage (NeuAcα2,3Gal and NeuGcα2,3Gal), agglutinated erythrocytes from all of the animal species tested (chickens, ducks, guinea pigs, humans, sheep, horses, and cows). The human viruses, including those known to preferentially recognize NeuAcα2,6Gal, agglutinated all but the horse and cow erythrocytes. Fluorescence-activated cell sorting analysis of erythrocytes using linkage-specific lectins [*Sambucus nigra* agglutinin for sialic acid (SA)α2,6Gal and *Maackia amurensis* agglutinin for SAα2,3Gal] showed that both cow and horse erythrocytes contain a large amount of SAα2,3Gal-, but virtually no SA2,6Gal-specific lectin-reactive oligosaccharides on the cell surface, while human and chicken erythrocytes contained both types of oligosaccharides. Considering that the majority (>93%) of sialic acid in horse and cow erythrocytes is of the *N*-glycolyl type, our results suggest that viruses able to agglutinate these erythrocytes (i.e., avian and equine viruses) recognize NeuGcα2,3Gal. These findings also show that agglutinating assays with erythrocytes from different animal species would be useful in characterizing the receptor specificity of influenza A viruses.

Since its recognition by Hirst in 1941 (*10*), the ability of human influenza A and B viruses to agglutinate the erythrocytes of different animal species has been studied (*13*). A change in hemagglutination during the passage of human viruses in eggs was first reported as O-D (original-derived) variation. O-form viruses agglutinate human or guinea pig erythrocytes in preference to chicken erythrocytes, while D-form viruses agglutinate all three types equally (*3*). Influenza C viruses, containing a single-envelope hemagglutinin-esterase glycoprotein and recognizing 9-*O*-acetyl neuraminic acid (*8, 24, 33*), agglutinate chicken, mouse, rat, and frog erythrocytes, but not others, including those from humans, guinea pigs, cows, pigs, and horses (*34*). These findings suggest that the receptor specificity of influenza viruses is related to the agglutination of erythrocytes from different animal species; however, the molecular basis for agglutination of erythrocytes has not been resolved.

[1] To whom correspondence and reprint requests should be addressed at current address: Department of Veterinary Public Health, Faculty of Agriculture, Tottori University, Tottori 680, Japan. Fax:(+81-857)31-5436. E-mail: toshiito@agr.tottori-u.ac.jp.

Influenza A viruses bind to cell surface sialyloligosaccharides with specificities that vary according to the host species of origin (*22*). Human viruses preferentially bind to oligosaccharides containing *N*-acetylneuraminic acidα2,6-galactose (NeuAcα2,6Gal), while avian and equine influenza strains bind to NeuAcα2,3Gal. A single amino acid change in the hemagglutinin selected with a horse serum inhibitor, α_2-macroglobulin, altered the receptor specificity of a human influenza virus [A/Aichi/2/68 (H3N2)] from NeuAcα2,6Gal to NeuAcα2,3Gal (*23*).

In previous studies, the receptor specificity of influenza A and B viruses was determined by direct binding assays based on different gangliosides (*4, 31*), hemagglutination with erythrocytes enzymatically modified to contain sialyloligosaccharides of defined sequences (*22*), or hemolysis assays based on ganglioside-coated erythrocytes (*30*). These methods require specific reagents (e.g., gangliosides, sialyltransferases specific for different sialic acid(SA)-galactose linkages or different sialic acids, CMP-NeuAc, CMP-NeuGc, and CMP-9-*O*-Ac-NeuAc) and tend to be technically difficult and time-consuming.

Because the oligosaccharide composition of glycopro-

0042-6822/97 $25.00

TABLE 1

Hemagglutinating Activity of Representative Human, Avian, and Equine Influenza Viruses with Erythrocytes from Different Animal Species[a]

Virus	Hemagglutination with erythrocytes from[b]			
	Cow	Horse	Pig	Human
A/Singapore/57 (H2N2)	<2	<2	16	16
A/Aichi/2/68 (H3N2)	<2	<2	<2	8
A/Udorn/307/72 (H3N2)	<2	<2	<2	16
A/Memphis/102/72 (H3N2)	<2	<2	16	8
A/mallard/NY/6750/78 (H2N2)	16	16	8	16
A/duck/Hokkaido/8/80 (H3N8)	32	8	8	16
A/swine/Netherlands/3/80 (H1N1)	8	16	8	8
A/equine/Prague/1/56 (H7N7)	32	16	16	16
A/equine/Miami/1/63 (H3N8)	16	16	8	16

[a] Glass-slide hemagglutination tests were done with serial dilutions of viruses in allantoic fluid containing 256 HAU/ml, as determined with chicken erythrocytes in a microtiter assay (*28*).

[b] Titers are expressed as the reciprocal of the highest virus dilution producing large aggregates [see Fig. 1 for examples with A/Aichi/2/68 (H3N2) and A/duck/Hokkaido/8/80 (H3N8)].

teins and glycolipids differs among erythrocytes depending on the species of animal studied (*17, 36*), we thought that the ability of viruses to agglutinate erythrocytes might correlate with their receptor specificity. Here, we systematically analyzed the agglutination patterns of influenza A viruses isolated from different animal species, many with known receptor specificities, and used linkage-specific lectins (i.e., those recognizing SAα2,3-Gal or SAα2,6Gal on the erythrocyte surface) to identify the molecular basis for any observed differences in hemagglutination.

The influenza A viruses we used (Table 1) are maintained in repositories at the Laboratory of Microbiology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University and the Department of Virology and Molecular Biology, St. Jude Children's Research Hospital. They were grown in 11-day-old embryonated chicken eggs and used for hemagglutination, either with or without purification by differential centrifugation and sedimentation through a sucrose gradient (*18*).

Serum inhibitor-resistant mutants of A/Aichi/2/68 (H3N2) and A/Tokyo/6/73 (H3N2) were isolated in the presence of horse or guinea pig serum as described previously (*25*). Briefly, the viruses were incubated with equal volumes of undiluted serum at room temperature for 1 hr and then inoculated into 11-day-old embryonated chicken eggs. Mutants were isolated at limiting dilution, and their inhibitor resistance was verified by hemagglutination inhibition (HI) tests (*21*). Viral RNA was isolated from A/Aichi/2/68 (H3N2) and A/Tokyo/6/73 as previously described (*2*). cDNA was synthesized with reverse transcriptase and random hexamers (*16*). Products of the polymerase chain reaction (PCR) were directly sequenced with an autosequencer (Applied Biosystem Inc., Foster City, CA) according to the manufacturer's protocol. The sequence of the primer oligonucleotides used will be provided on request to the authors.

Erythrocytes from different animal species differ in their oligosaccharide composition. For example, those from chickens and humans contain only *N*-acetyl sialic acid (NeuAc), whereas those from horses and cows contain mainly *N*-glycolyl sialic acid (NeuGc, 100% for horses and 93% for cows; Ref. *30*). In addition, hematoside [II^3(Neu5Gc)LacCer; NeuGcα2,3βGal1,4GlcNAcβ1Cer], which contains NeuGcα2,3Gal, is the major ganglioside in equine (*7, 30*) but not human erythrocytes. These findings prompted us to screen viruses with defined receptor specificities for their ability to agglutinate erythrocytes from different animal species. With some mammalian erythrocytes, it was difficult to analyze the extent of hemagglutination using microtiter plates (data not shown), as these cells, unlike avian erythrocytes, do not possess a nucleus and hence do not readily sediment. We, therefore, substituted a glass-slide assay (Fig. 1A). The hemagglutinating activity was also quantified with the use of serially diluted viruses containing 256 HAU/ml (Fig. 1B). The results indicated considerable variation in the agglutination of different animal erythrocytes by the test viruses (Fig. 1B and Table 1). This variability was not affected by the purity of virus preparations, and differences remained constant so long as the hemagglutination titers of virus suspensions remained at 1:256 (determined with chicken erythrocytes in a microtiter assay; Ref. *28*). Hence, we screened a large number of viruses for their hemagglutinating activity using this virus concentration.

The A/Aichi/2/68 (H3N2) strain of human influenza virus, known to preferentially recognize NeuAcα2,6Gal (*22*), agglutinated chicken, duck, guinea pig, and human (type O) erythrocytes, but not those from cows, horses, and pigs (Table 2). By contrast, equine [A/equine/Miami/1/63 (H3N8)] and avian [A/duck/Ukraine/63 (H3N8) and A/mallard/New York/6750/78 (H2N2)] viruses, which preferentially recognize both NeuAcα2,3Gal and NeuGcα2,3Gal but not NeuAcα2,6Gal (9; Y. Suzuki, unpublished data), agglutinated erythrocytes from all sources. These findings indicate that agglutination of erythrocytes from cows, horses, and pigs requires NeuAcα2,3Gal or NeuGcα2,3-Gal recognition.

By enzymatically modifying human erythrocytes to contain the NeuGcα2,6Gal linkage, Higa *et al.* (*9*) demonstrated an evolutionary change in the type of sialic acid recognized by human H3 influenza viruses. That is, H3 human viruses isolated in 1968 recognize only NeuAc, whereas those isolated after 1972 recognize both NeuAc and NeuGc. We therefore sought a corresponding change in receptor specificity in human as well as avian and equine viruses by agglutination of erythrocytes from different animal species. Although the hemagglutination pat-

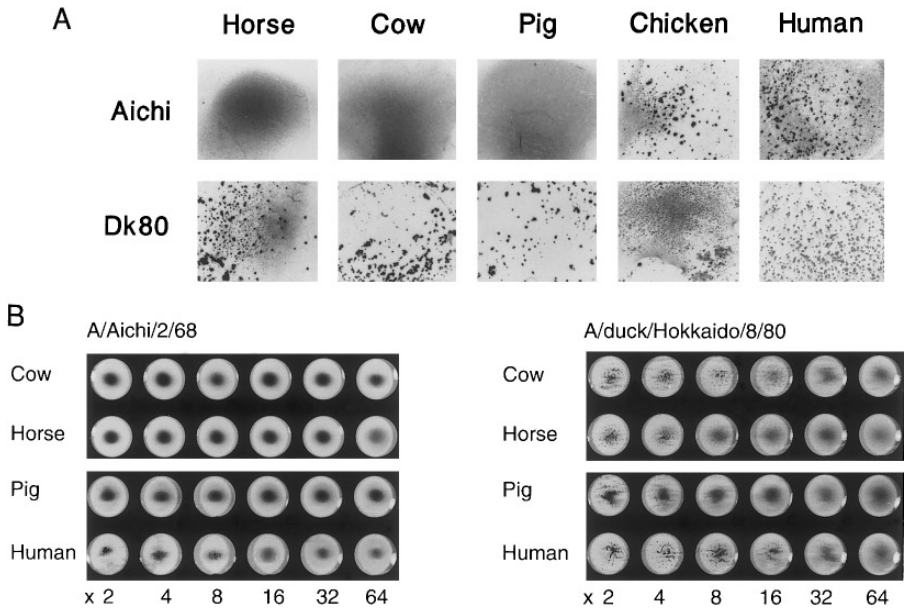

FIG. 1. Agglutination of erythrocytes from different animal species by influenza A viruses. Erythrocytes from different animal species were collected by venipuncture, stored at 4° as a 50% (v/v) suspension in Alsever's solution (*15*), and washed three times in phosphate-buffered saline (PBS, pH 7.2) before use. (A) Horse, cow, pig, and chicken erythrocytes [1.5% (v/v), 10 μl] were mixed with virus suspension (10 μl of 256 HAU/ml, as determined with chicken erythrocytes in a microtiter assay (*28*), and observed with the naked eye after 5 min of incubation at 0°. (B) The same viruses, at the same HA titer, were serially diluted twofold and examined for hemagglutination as described above.

terns observed among avian and equine viruses did not differ from each other, human H3 viruses isolated since but not before 1973 agglutinated pig erythrocytes. Human viruses isolated in 1972 were heterogenous in their ability to agglutinate pig erythrocytes. H2 human viruses isolated in 1957 also agglutinated pig erythrocytes. Because the human virus H2 HA was introduced from birds in 1957 (*19, 27*), these findings suggest that the early human H2 HAs retain some characteristics of avian viruses. They also suggest that agglutination of pig erythrocytes requires recognition of NeuGc.

Swine viruses, in contrast to avian, equine and human viruses, differed from each other in their ability to agglutinate erythrocytes (Table 2). Avian-like A/swine/Netherlands/3/80 (H1N1) and human-like A/swine/Colorado/77 (H3N2) agglutinated all of the erythrocytes tested, while other swine viruses agglutinated only chicken, duck, human, and guinea pig erythrocytes.

Bovine and equine erythrocytes, known to contain mainly NeuGc (*30*), were agglutinated by avian and equine but not human viruses isolated after 1973, even though the latter recognize NeuGc (*9*). Thus, the α2,3 linkage, in addition to NeuGc recognition, appears essential for agglutination of bovine and equine erythrocytes. To test this prediction, we made A/Aichi/2/68 and A/Tokyo/6/73 viruses horse serum inhibitor-resistant, which should convert their specificity from the α2,6 to a α2,3 linkage (*23*). Direct binding assays with human transferrin containing only NeuAcα2,6Gal (*14, 29*) and sialosylparagloboside, [IV^3(Neu5Ac)nLc4Cer and IV^3(Neu5Gc)nLc4Cer], verified that both of the resistant viruses preferred the α2,3 linkage (Table 3). Moreover, the A/Tokyo/6/73 but not the A/Aichi/2/68 variant bound to IV3(Neu5Gc)nLc4Cer, indicating its ability to recognize NeuGc. Horse serum-resistant A/Tokyo/6/73, unlike its parent or horse serum-resistant A/Aichi/2/68, agglutinated erythrocytes from all of the animal species tested (Table 3). These results suggest that agglutination of cow and horse erythrocytes by influenza virus requires NeuGcα2,3Gal recognition. They also indicate that recognition of the α2,3 linkage and *N*-glycolyl group of sialic acid is determined by different amino acid residues in the HA molecule.

To understand the structural basis of horse serum resistance in the A/Aichi/2/68 and A/Tokyo/6/73 HA viruses, we determined the nucleotide sequences corresponding to the HA1 region and deduced the amino acid sequences. Both the A/Aichi/2/68 and A/Tokyo/6/73 serum-resistant viruses had a single mutation at residue 226 from Leu to Glu, as previously reported (*23*).

How does one account for animal species-specific hemagglutination by different influenza viruses? To address this question, we analyzed the relative abundance of SAα2,3Gal and SAα2,6Gal linkages on erythrocytes of different animal species by fluorescence-activated cell sorting using linkage-specific lectins. Human and chicken erythrocytes that were agglutinated by a variety of viruses contained both SAα2,3Gal and SAα2,6Gal linkages (Fig. 2). Although pig erythrocytes contained both types of linkages, SAα2,6Gal was more abundant than SAα2,3Gal. By contrast, horse and cow erythrocytes, which were agglutinated by only avian and equine viruses among natural isolates, contained mainly the SAα-

TABLE 2

Glass-Slide Hemagglutination of a Variety of Human, Avian, Swine, and Equine H3 Influenza Viruses with Erythrocytes from Different Animal Species[a]

Virus	Hemagglutination with erythrocytes from			
	Cow	Horse	Pig	Chicken duck human guinea pig
Human isolates				
A/Puerto Rico/8/34 (H1N1)	−	−	−	+
A/Fort Mommouth/1/47 (H1N1)[b]	−	−	−	+
A/Singapore/57 (H2N2)	−	−	+	+
A/Japan/305/57 (H2N2)	−	−	+	+
A/Aichi/2/68 (H3N2)	−	−	−	+
A/Fukuoka/1/70 (H3N2)[b]	−	−	−	+
A/Chiba/5/71 (H3N2)[b]	−	−	−	+
A/Kumamoto/1/72 (H3N2)	−	−	−	+
A/Tokyo/1/72 (H3N2)	−	−	−	+
A/England/42/72 (H3N2)	−	−	−	+
A/Udorn/307/72 (H3N2)	−	−	−	+
A/Memphis/101/72 (H3N2)	−	−	+	+
A/Memphis/102/72 (H3N2)	−	−	+	+
A/Tokyo/6/73 (H3N2)	−	−	+	+
A/Victoria/3/75 (H3N2)	−	−	+	+
A/Kumamoto/22/76 (H3N2)	−	−	+	+
A/Texas/1/77 (H3N2)	−	−	+	+
A/Yamanashi/2/77 (H3N2)	−	−	+	+
A/Niigata/102/81 (H3N2)[b]	−	−	+	+
A/Los Angeles/2/87 (H3N2)	−	−	+	+
Avian isolates				
A/chicken/Brescia/02 (H7N1)[b]	+	+	+	+
A/FPV/Rostock/34 (H7N1)[b]	+	+	+	+
A/duck/Ukraine/1/63 (H3N8)	+	+	+	+
A/turkey/Ontalio/7732/66 (H5N9)[b]	+	+	+	+
A/duck/Hokkaido/5/77 (H3N2)	+	+	+	+
A/myna/Tokyo/7/77 (H3N2)	+	+	+	+
A/myna/Tokyo/9/77 (H3N2)	+	+	+	+
A/mallard/NY/6750/78 (H2N2)	+	+	+	+
A/mallard/NY/6874/78 (H3N4)	+	+	+	+
A/budgerigar/Aichi/79 (H3N2)	+	+	+	+
A/duck/Hokkaido/8/80 (H3N8)	+	+	+	+
A/duck/Hokkaido/16/80 (H3N8)	+	+	+	+
A/duck/Hokkaido/33/80 (H3N8)	+	+	+	+
A/gull/Massachusetts/26/80 (H13N6)	+	+	+	+
A/duck/Hokkaido/7/82 (H3N8)	+	+	+	+
A/duck/Ireland/83 (H5N8)[b]	+	+	+	+
A/chicken/Victoria/85 (H7N7)[b]	+	+	+	+
A/ruddy turnstone/New Jersey/47/85 (H4N6)	+	+	+	+
A/mallard/Alberta/7/89 (H9N3)	+	+	+	+
A/mallard/Alberta/58/89 (H8N4)	+	+	+	+
A/turkey/Minnesota/12537/89 (H1N1)	+	+	+	+
A/mallard/Alberta/11/91 (H6N4)	+	+	+	+
A/turkey/England/91 (H7N7)[b]	+	+	+	+
A/mallard/Alberta/24/92 (H11N9)	+	+	+	+
A/chicken/Mexico/31381/94 (H1N1)	+	+	+	+
A/duck/Australia/749/80 (H1N1)	+	+	+	+
Swine isolates				
Classic				
A/swine/Iowa/15/30 (H1N1)	−	−	−	+
A/swine/Hokkaido/2/81 (H1N1)	−	−	−	+
Avian-like				
A/swine/Netherlands/3/80 (H1N1)	+	+	+	+

TABLE 2—*Continued*

Virus	Hemagglutination with erythrocytes from: Cow	Horse	Pig	Chicken duck human guinea pig
Human-like				
A/swine/Colorado/77 (H3N2)	+	+	+	+
A/swine/Italy/309/83 (H3N2)	−	−	−	+
A/swine/Italy/526/85 (H3N2)	−	−	−	+
A/swine/Italy/635/87 (H3N2)	−	−	−	+
Equine isolates				
A/equine/Prague/1/56 (H7N7)	+	+	+	+
A/equine/Miami/1/63 (H3N8)	+	+	+	+
A/equine/Detroit/3/64 (H7N7)	+	+	+	+
A/equine/tokyo/2/71 (H3N8)	+	+	+	+
A/equine/London/1416/73 (H7N7)	+	+	+	+
A/equine/New York/49/73 (H7N7)	+	+	+	+
A/equine/Kentucky/75 (H7N7)	+	+	+	+
A/equine/Kentucky/1/76 (H3N8)	+	+	+	+
A/equine/Kentucky/77 (H7N7)	+	+	+	+
A/equine/Cordoba/4/76 (H7N7)	+	+	+	+
A/equine/Fontainbleau/79 (H3N8)	+	+	+	+
A/equine/Kentucky/1/81 (H3N8)	+	+	+	+
A/equine/Tennessee/5/86 (H3N8)	+	+	+	+
A/equine/Kentucky/92 (H3N8)	+	+	+	+

[a] +, Agglutination; −, no agglutination with viruses containing 256 HAU/ml, as determined with chicken erythrocytes in a microtiter assay (*28*).
[b] Virus-containing allantoic fluid was used; the other viruses were tested after purification (*18*).

2,3Gal linkage. These findings indicate that the relative abundance of SAα2,3Gal or SAα2,6Gal linkages on erythrocytes determines whether or not the cells will be agglutinated by viruses with specific linkage preferences. Considering that the vast majority of sialic acid on horse and cow erythrocytes (100 and 93%, respectively) is NeuGc, agglutination of these cells probably requires NeuGcα2,-3Gal recognition.

Here we show that the ability of influenza A viruses to agglutinate erythrocytes from different animal species correlates with their receptor specificity. Therefore, erythrocytes from different hosts can be used to rapidly define the receptor specificity of influenza A viruses. Hemagglutination of cow and horse erythrocytes was associated with NeuGcα2,3Gal recognition. Inhibitor-resistant variants of the A/Aichi/2/68 strain, which preferentially recognize NeuAcα2,-3Gal but not NeuGcα2,3Gal, failed to agglutinate cow and horse erythrocytes, consistent with the limited amount of NeuAc on the surface of these cells (*30*). Fluorescence-activated cell sorting analysis of cow and horse erythrocytes, using fluorescence-conjugated linkage specific lectins, confirmed the abundance of SAα2,3Gal linkages on these erythrocytes. Thus, NeuGcα2,3Gal may be the dominant sialyloligosaccharide on the surface of cow and horse erythrocytes.

Pig erythrocytes were agglutinated by all avian and equine viruses tested as well as human strains isolated after 1973. Sixty-six percent of the sialic acid in pig erythrocytes is of the *N*-glycolyl type (*6*). These findings, together with the linkage specificity of human (α2,6) and avian and equine (α2,3) viruses, suggest that both NeuGcα2,3Gal and NeuGcα2,6Gal are present on the surface of pig erythrocytes, a prediction supported by the results of lectin assays (Fig. 2).

An avian-like swine virus (A/swine/Netherlands/3/80), which was introduced into European pigs in 1979 (*26*), agglutinated all of the erythrocytes tested. This property may reflect the retention of avian-like receptor specificities during their replication in pigs, since all the avian viruses we examined showed the same hemagglutinating activities. However, one of the human-like swine viruses (A/swine/Colorado/77) also agglutinated each of the erythrocytes tested, whereas other swine viruses did not (Table 1). Direct analysis of receptor specificity using defined glycoconjugates is needed to understand the structural basis of this variation.

Analysis of the three-dimensional structure of the H3 influenza virus HA complexed with sialyllactose, a cell receptor analogue, suggests that substitution of Tyr for Thr at residue 155 in human H3 strains isolated after 1972 was responsible for the acquisition of NeuGc recognition capacity (*35*), since the methyl group in NeuGc faces toward amino acid 155 of the hemagglutinin and the Thr–Tyr mutation increases the affinity for NeuGc. The HA sequences of the human viruses used in the present study generally support the notion that NeuGc recognition enables human H3 viruses to agglutinate pig erythrocytes. However, the A/Udorn/307/72 (H3N2) and

TABLE 3

Receptor Specificity of Parental and Inhibitor-Resistant Variants of Human Influenza Viruses

Virus	Hemagglutination with erythrocytes from: Cow horse	Pig	Guinea pig chicken duck human	Binding to: Transferrin (NeuAcα2,6Gal)	Sia-PG[a] (NeuAcα2,3Gal)	Sia-PG (NeuGcα2,3Gal)
A/Aichi/2/68						
Parent	−	−	+	+	−	−
HSIR[b]	−	−	+	−	+	−
GPSIR[c]	−	−	+	−	+	−
A/Tokyo/6/73						
Parent	−	+	+	+	−	−
HSIR	+	+	+	−	+	+

Note. Direct binding assays of viruses to gangliosides or sialoglycoprotein (human transferrin) were performed by a modification of the method of Holmgren *et al.* (11). Briefly, 5 μ*M* ganglioside or human transferrin in PBS (5 μl) was added to tissue culture dishes (6-cm diameter; Coaster Co., MA), which were rinsed with PBS after overnight incubation at room temperature in a humid atmosphere. Unoccupied binding sites were blocked by incubation with gelatin (10 mg/ml) in PBS for 30 min. The dishes were then rinsed with PBS, allowed to incubate for 1 hr on ice with 3 ml of virus (>512 HAU/ml) in PBS, and subsequently rinsed three times with cold PBS. After addition of 5 ml of a 1% suspension of chicken erythrocytes in PBS and 15 min of incubation on ice, unadsorbed erythrocytes were washed from the dishes and binding of the virus to glycoconjugates was determined by hemadsorption.

[a] Sialosylparaglobosides [IV^3(Neu5Ac)nLc4Cer(NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ 1 Cer)] from human erythrocytes were purified at the Department of Biochemistry, School of Pharmaceutical Science, University of Shizuoka. IV^3(Neu5Gc)nLc4Cer (NeuGcα2,3Galβ1,4GlcNAcβ1,3-Galβ1,4Glcβ 1 Cer) from equine erythrocytes was generously provided by Dr. Masaharu Naiki, National Institute of Health, Japan. Human transferrin was purchased from Midori Cross Co. Ltd. (Osaka, Japan).

[b] Horse serum inhibitor-resistant variant.

[c] Guinea pig serum inhibitor-resistant variant.

A/England/42/72 (H3N2) strains contain Tyr at position 155 but do not agglutinate pig erythrocytes. Thus, recognition of other residues may be important in the agglutination of pig erythrocytes by human H3 viruses isolated after 1973; further studies are needed to clarify this issue.

Recently, Conner *et al.* (*5*) suggested the importance of an amino acid change at residue 228 of the H3 HA in addition to that at residue 226 (*23*) for the receptor specificity; however, this study was restricted to the α2,3 versus α2,6 linkage, excluding NeuGc versus NeuAc. Although crystallographic data pertinent to interactions of NeuGcα2,3Gal with the HAs of nonhuman virus strains are still unavailable, amino acid substitutions at residue 228 could increase the affinity for NeuGc, since the majority of avian and equine virus HAs agglutinate cow and horse erythrocytes (hence recognize NeuGc) and have Gly at position 228, whereas the HAs of human H3 viruses contain Ser at this position.

Early studies of the agglutination of erythrocytes from different animal species yielded inconsistent results (*13*),

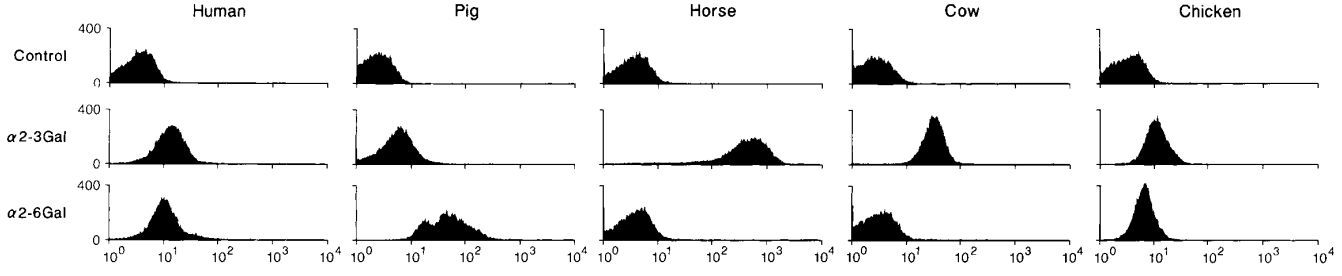

FIG. 2. Comparison of the relative amounts of SAα2,3Gal and SAα2,6Gal linkages on the surface of animal erythrocytes. For detection of SAα2,3Gal and SAα2,6Gal on the surface of erythrocytes, a digoxigenin (DIG) glycan differentiation kit (Boehringer Mannheim Biochemicals, Inc.) was used. Briefly, cow, pig, horse, chicken, and human (type O) erythrocytes were washed twice in PBS containing 10 m*M* glycine, then once with Buffer 1 (50 m*M* Tris–HCl, 0.15 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 7.5). A blocking solution supplied with the DIG kit was then incubated with the cells for 1 hr, after which the cells were washed as described above. DIG-labeled lectins [*Sambucus nigra* agglutinin (SNA) specific for SAα2,6Gal and *Maackia amurensis* agglutinin (MAA) specific for SAα2,3Gal] dissolved in Buffer 1 were then incubated with the cells for 1 hr. After three washes, the cells were incubated with anti-DIG antibody conjugated to fluorescein isothiocyanate (Fab fragments) for 1 hr. After an additional three washes, they were analyzed for fluorescence intensity on a FACScan fluorospectrometer (Becton Dickinson). The profile shown depicts cell number as a function of log fluorescence intensity of SAα2,3Gal (α2,3; *Maackia amurensis* agglutinin)- and SAα2,6Gal (α2,6; *Sambucus nigra* agglutinin)-specific lectin-reactive oligosaccharide on the cell surface.

probably because of differences in the temperatures at which experiments were performed (*12, 20, 32*). We were able to hold experimental variability to a minimum in the present study by conducting all tests at 0°, thus eliminating the untoward effects of the viral neuraminidase activity. Another modification that led to better reproducibility of test results was the substitution of glass slides for microtiter plates. Although used routinely in assessing hemagglutination by influenza viruses, microtiter plates had major disadvantages in our hands. It was difficult, for example, to analyze the extent of hemagglutination with mammalian erythrocytes, which unlike avian erythrocytes do not possess a nucleus and therefore do not readily sediment. Even the use of microtiter plates with V-shaped wells and erythrocyte suspensions containing 0.5% BSA was inadequate to correct this problem. By contrast, hemagglutination tests on glass slides yielded highly reproducible results whether avian or mammalian cells were used.

It is well established that avian and equine viruses preferentially recognize SAα2,3Gal, while human viruses bind to SAα2,6Gal (*22*). The presence of SAα2,6Gal but not SAα2,3Gal on the surface of epithelial cells in human trachea (*1*) underscores the importance of the role of receptor specificity (i.e., types of linkages between SA and Gal) in the host range restriction of influenza viruses. Whether different types of sialic acids also influence the host range has not been investigated to any appreciable extent. The present data suggest that the recognition of NeuGc may also be important for host range restriction of influenza viruses.

ACKNOWLEDGMENTS

We thank Krisna Wells for excellent technical assistance, Dr. Masaharu Naiki for IV^3(Neu5Gc)nLc4Cer, Richard Ashman for assistance with the FACS analysis, and John Gilbert for editing the manuscript. This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan, and by Public Health Service Research Grant AI33898 from the National Institute of Allergy and Infectious Disease, by a Cancer Center Support (CORE) grant, and by the American Lebanese Syrian Associated Charities (ALSAC).

REFERENCES

1. Baum, L. G., and Paulson, J. C. *Acta Histochem.* Suppl.-Band XL, S. 35–38, (1990).
2. Bean, W. J., Sriram, G., and Webster, R. G. *Anal. Biochem.* 102, 228–232, (1980).
3. Burnet, F. M., and Bull, D. R. *Aust. J. Exp. Biol. Med. Sci.* 21, 55–69, (1941).
4. Carrol, S. M., Higa, H. H., and Paulson, J. C. *J. Biol. Chem.* 256, 8357–8363, (1981).
5. Conner, R. J., Kawaoka, Y., Webster, R. G., and Paulson, J. C. *Virology* 205, 17–23, (1994).
6. Eylar, E. H., Madoff, M. A., Brody, O. V., and Oncley, J. L. *J. Biol.-Chem.* 237, 1992–2000, (1962).
7. Gasa, S., Makita, A., and Kinoshita, Y. *J. Biol. Chem.* 258, 876–881, (1983).
8. Herrler, G., and Klenk, H. -D. *Virology* 159, 102–108, (1987).
9. Higa, H. H., Rogers, G. N., and Paulson, J. C. *Virology* 144, 279–282, (1985).
10. Hirst, G. K. *Science* 94, 22–23, (1941).
11. Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P., and Strannegard, O. *Proc. Natl. Acad. Sci. USA* 77, 1947–1950, (1980).
12. Horvath, I., Szollosy, E., and Ivanovics, G. *Acta Physiol. Hung.* 2, 77–86, (1950).
13. Hoyle, L. (1968). *In* "Virology Monographs," No. 4, p. 88, Springer-Verlag, New York/Berlin.
14. Jamieson, G. A., Jett, M., and DeBernardo, S. L. *J. Biol. Chem.* 246, 3686–3693, (1971).
15. Joseph, J. M. *In* "Gradwohl's Clinical Laboratory Methods and Diagnosis" (A. C. Sonnenwirth and L. Jarett, Eds.), Vol. 2, pp. 2034–2048, C. V. Mosby Co., St. Louis/Toronto/London, 1980.
16. Katz, J. M., Wang, M., and Webster, R. G. *J. Virol.* 64, 1808–1811, (1990).
17. Klenk, E., and Uhlenbruck, G. *Hoppe-Seyl. Z.* 311, 227–233, (1958).
18. Laver, W. G. *In* "Fundamental Techniques in Virology" (K. Habel and N. P. Salzman, Eds.), pp. 82–86. Academic Press, New York, 1969.
19. Laver, W. G. and Webster, R. G. *Virology* 51, 383–391, (1973).
20. Overman, J. R., and Friedewald, W. F. *J. Immunol.* 62, 415–424, (1949).
21. Palmer, D. F., Coleman, M. T., Dowdle, W. R., and Schild, G. C. *In* "Immunology Series," No. 6, pp. 51–52, U.S. Department of Health, Education, and Welfare, Washington, DC, 1975.
22. Rogers, G. N., and Paulson, J. C. *Virology* 127, 361–373, (1983).
23. Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. *Nature* 304, 76–78, (1983).
24. Rogers, G. N., Herrler, G., Paulson, J. C., and Klenk, H. -D. *J. Biol. Chem.* 261, 5947–5951, (1986).
25. Ryan-Poirier, K. A., and Kawaoka, Y. *J. Virol.* 65, 389–395, (1991).
26. Scholtissek, C., Burger, H., Bachmann, P. A., and Hannoun, C. *Virology* 129, 521–523, (1983).
27. Scholtissek, C., Rohde, W., Von Hoyningen, V., and Rott, R. *Virology* 87, 13–20, (1978).
28. Sever, J. L. *J. Immunol.* 88, 320–329, (1962).
29. Spik, G., Bayard, B., Fournet, B., Strecker, G., Bouquelet, S., and Montreuil, J. *FEBS Lett.* 50, 296–299, (1975).
30. Suzuki, Y., Matsunaga, M., and Matsumoto, M. *J. Biol. Chem.* 260, 1362–1365, (1985).
31. Suzuki, Y., Nakao, T., Ito, T., Watanabe, N., Toda, Y., Guiyun, X., Suzuki, T., Kobayashi, T., Kimura, Y., Yamada, A., Sugawara, K., Nishimura, H., Kitame, F., Nakamura, K., Deya, E., Kiso, M., and Hasegawa, A. *Virology* 189, 121–131, (1992).
32. Tamm, I. *J. Immunol.* 73, 180–189, (1954).
33. Vlasak, R., Krystal, M., Nacht, M., and Palese, P. *Virology* 160, 419–425, (1987).
34. Wang, S.-P., and Lin, T.-Y. *J. Infect. Dis.* 103, 178–182, (1958).
35. Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. *Nature* 333, 426–431, (1988).
36. Yamakawa, T., and Nagai, Y. *Trends Biochem. Sci.* 3, 128–131, (1978).